(12) UK Patent Application (19) GB (11) 2 037 811 A

(21) Application No 7940147
(22) Date of filing 20 Nov 1979
(30) Priority data
(31) 783555
(32) 21 Nov 1978
(33) Finland (FI)
(43) Application published
16 Jul 1980
(51) INT CL³
C12M 1/00 // 1/22
(52) Domestic classification
C6F CE
(56) Documents cited
GB 1524401
GB 1502674
GB 1472981
GB 1437404
GB 1408180
GB 1390766
GB 1345429
GB 1248197
GB 1242466
GB 1132455
(58) Field of search
C6F
(71) Applicants
Orion-yhtymä Oy,
Nilsiänkatu 10-14, SF-00510 Helsinki 51, Finland
(72) Inventor
Jouko Savolainen
(74) Agents
J. Y. & G. W. Johnson

(54) Identification of Microorganisms

(57) A kit for the identification of microorganisms, is made up of a plate 10 subdivided into several compartments A, B at least some of which B contain a solid medium 13 on which a microorganism can grow after the addition of one or more nutrients and at least one of which A preferably contains a solid medium containing all the nutrients necessary for microorganism growth, and a variety of different solid tablets supplying the nutrient(s) lacking from the media in the compartments B in a variety of ways. The morphology of the microorganism to be identified is studied in compartment A and its metabolism when presented with a variety of different candidate nutrients, usually in the tablets, is studied in the compartments B, these compartments preferably being used merely to study carbohydrate and nitrogen metabolism and its reaction to the urease test.

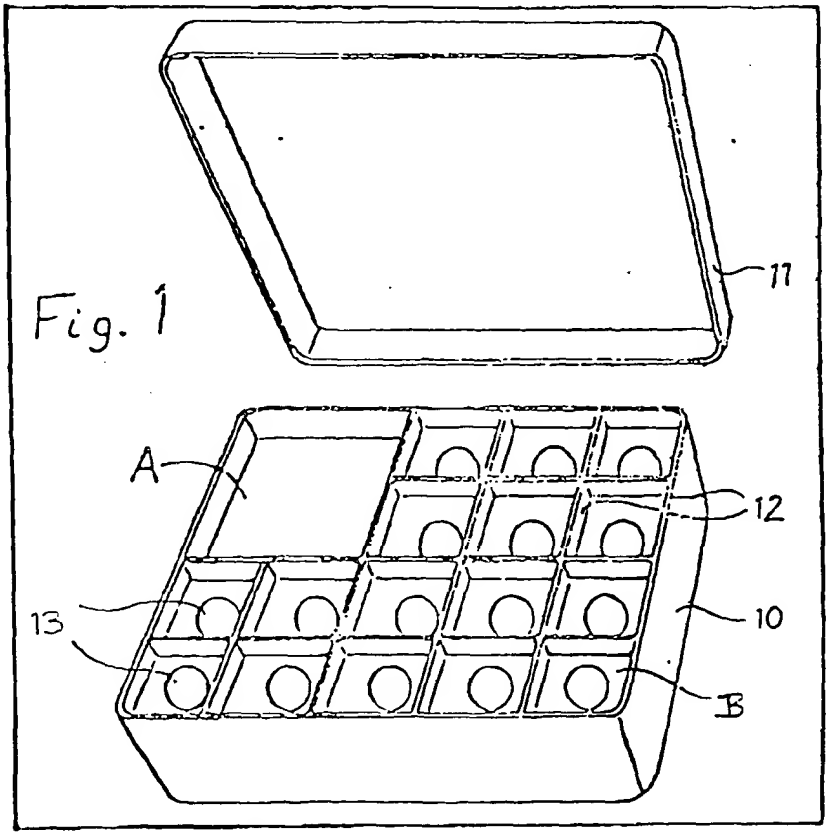

Fig. 1

GB 2 037 811 A

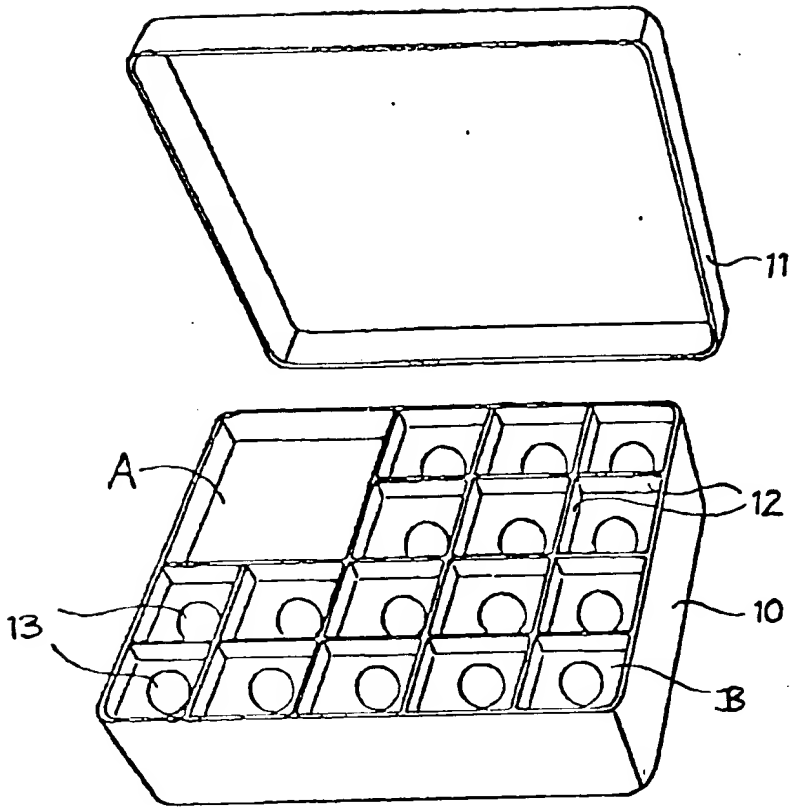

Fig. 1

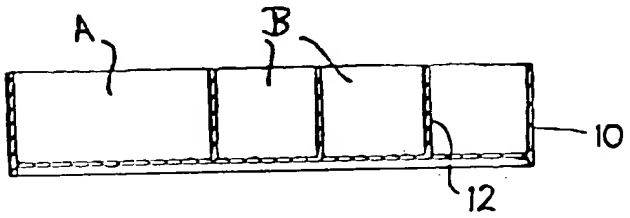

Fig. 2

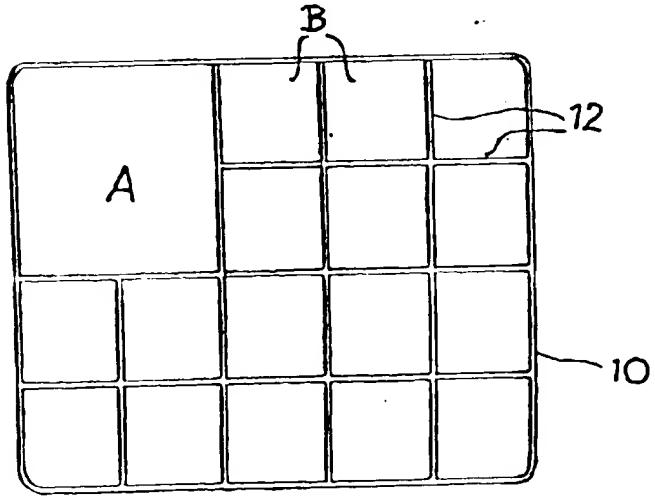

Fig. 3

SPECIFICATION
Identification of Microorganisms

This invention relates to the identification of microorganisms by investigating their carbohydrate and nitrogen metabolism and their morphology. By microorganisms, otherwise referred to as microbes, we mean primarily actinomycete, yeasts and bacteria.

In human and veterinary medicine as well as in the food and fermentation industries the identification of microorganisms is important. Knowledge of the identity of a microbe is indispensable in choosing a method for its destruction or for modification of its growth conditions. The identification of pathogenic microorganisms is essential in assessing their pathogenicity and selecting the right therapy.

Microorganisms are useful in the food and fermentation industry, but they are also often contaminative. These contaminants must be identified so that their origin can be found and the best way to prevent their further growth and spreading be selected.

The identification of microbes is based principally on their morphology, their ability to utilize various carbohydrates and nitrogen sources, their growth condition requirements, and some other characteristics.

Carbohydrate assimilation tests and nitrogen metabolism tests can be performed according to any of three fundamentally different methods: the auxanographic method, the broth tube method of Wickerham, and the slant tube method developed from the latter. They are described in the Manual of Clinical Microbiology, 2nd ed., 1974, pp. 481—507, and Journal of Clinical Microbiology 2, No. 1, 1975, pp. 21—34.

The methods mentioned above and their various modifications have certain drawbacks. They require much equipment—test tubes, plates, culture media, etc.—they take up much working space and they are laborious and time-consuming. Moreover, the risk of contamination increases with the phases of work and the amount of material used.

The present invention seeks to provide a means of avoiding or overcoming these disadvantages. We have found that it is possible to carry out all the necessary tests except fermentation tests, i.e. all the carbohydrate and nitrogen metabolism tests required for identification, as well as the cultivation for the morphological test, on a single plate or dish, which is divided into several compartments. Thus the material, time, and labour required, as well as the phases of work, can be essentially reduced.

According to the invention there is provided a device for use in the identification of microorganisms by studying their growth under different circumstances, comprising a plate subdivided into several isolated compartments at least some of which contain a solid medium on which a microorganism to be identified can grow after the addition of one or more nutrients, whereby the effect of adding different possible nutrients for microorganism metabolism can be studied.

The invention further provides a kit for use in the identification of microorganisms comprising a device as specified above and a plurality of tablets each containing one or more nutrients and/or candidate nutrients for the growth of the microorganism, optionally a binder and/or a filler and optionally an indicator for indicating the results of the study on the effect of adding a particular candidate nutrient.

The invention also provides a method of identifying microorganisms employing a kit as defined above, comprising inoculating the solid media in the compartments with the microorganism to be identified; studying the morphology of the microorganism growing in at least one of the compartments in the presence of all the necessary nutrients for its growth; adding the tablets to compartments containing solid media lacking one or more nutrients, the tablets being selected so that after their addition and the addition of any non-solid candidate nutrient each compartment (other than any desired control compartment) has all the necessary nutrients for microorganism growth except that in some compartments a candidate nutrient is present instead of a nutrient, a variety of different candidate nutrients being used in different compartments; and studying the effect on microorganism growth of the candidate nutrients.

One embodiment of a device according to the invention is shown in the accompanying drawings, in which

Figure 1 is a perspective view of the plate with culture media and tablets in position;

Figure 2 is a vertical cross-section of the plate; and

Figure 3 is a plan view of the plate from above.

Referring to the drawings, the device is a sterile, rectangular plate 10 provided with a cover 11 and divided into several square compartments, one of which A is larger than the others B, by impenetrable partitions 12 rising from the bottom of the plate 10 and crossing each other at right angles. The size and number of the compartments can be chosen at will. Compartment A is primarily intended for morphological studies, but may alternatively be used for other tests, whereas compartments B are primarily intended for metabolism tests. On the bottom of each compartment B there is a slightly buffered or unbuffered, solidified growth medium 13, e.g. an agar medium, suitable for each test. The agar medium B may or may not contain an indicator; alternatively, the indicator may be added after incubation.

In use of the plate, the agar medium is inoculated with a suitable amount of a suspension of the microorganism to be tested. After inoculation, any tablets containing nutrients necessary for the growth of the microorganisms to be tested, and pharmacologically approvable filling substances and binders, may be applied to

the agar medium with the aid of a dispenser, which may be of any known type suited to the plate.

The number and kind of tests may be varied as required for identification of the type of microorganism concerned. This is accomplished by varying the culture media used according to the invention and the tablets applied to these solidified media.

In testing yeasts, for instance, the culture medium in compartment A for morphological testing is suitably a corn meal agar of generally known type, but other media of low nutritional value may also be used. This is preferably a complete culture medium, usually the only complete culture medium in the identification system and does not require any tablet to be added. Suitably compartment A is of a size such that there is just room in the compartment for a cover-glass, so that microscopy can be performed directly in the plate and no separate preparations need be made for that purpose. To facilitate microscopy, known colouring agents may be added to the medium.

One or more of the compartments B are intended to be used for carbohydrate metabolism tests. The culture media for the carbohydrate tests and a control test generally consist of agar, an indicator and a buffer. As an indicator there may be used any pH-indicator sensitive to acid formation and decrease of pH below 5.5, e.g. bromcresol purple or bromthymol blue. The pH of the medium is adjusted to the middle of the colour range of the indicator, and the medium is slightly buffered against occasional variations of pH, e.g. with 5—10 mM phosphate buffer.

One or more of the other compartments B are intended to be used to test nitrogen metabolism, especially the ability to utilize nitrate and the ammonium ion in nitrogen metabolism. For this purpose a culture medium is generally used which contains agar and an indicator, for instance bromthymol blue, and the pH is conveniently adjusted to between blue and yellow, e.g. to pH 6.4.

A further test which can be performed in one or more of the compartments is the urease test. For this the culture medium generally contains agar and an indicator, e.g. phenol red and the pH is conveniently adjusted to 6.9.

We have found that the above-mentioned 4 tests are very often sufficient to provide full identification. Accordingly a preferred identification system requires only four different culture media, of which only one, the morphology medium, is complete. The other media lack nutrients; thus the growth of contaminants is effectively restrained.

Since only four different media are required, this identification method is much less laborious than other known methods, where different culture media must be prepared for each separate test. At the same time the stability of the media used in this system is better and the phases of work are fewer. An indicator need not necessarily be contained in the medium but can be added, e.g. pipetted, to the same after incubation.

Other necessary components of the test substrates—such as carbon source, nitrogen source, trace elements, and vitamins—are conveniently contained in tablets which, together with the medium in the plate, constitute the whole growth-maintaining system. Each tablet contains the nutrients necessary for the growth of the microorganisms to be tested (e.g. the components of the media used in Wickerham's broth tube method), the substance whose effect on the microorganism is to be tested, e.g. carbohydrate, and inert filler material. The latter may be any pharmacologically useable tablet-filler material which during cultivation releases the components required for growth onto the medium in the plate. Such materials are, for instance, cellulose and its derivatives. Binders, such as plastics products suitable for pharmacological purposes, may be used to improve the firmness of the tablets and facilitate their preparation.

A suitable composition for a tablet for testing carbohydrate metabolism in percentages by weight, is as follows: carbohydrate 12.7—25.4%, nutrients necessary for the growth of the microorganism—e.g. Difco Yeast Nitrogen Base—4.3%, filler material 60.6—74.5%, and binder 9.5%.

In the preparation of carbohydrate tablets various crystallizing solid monosaccharides, disaccharides, oligosaccharides and polysaccharides, or some other solid carbon sources, may conveniently be used as carbohydrate source.

For the control test in carbohydrate metabolism a tablet is prepared which contains all the components except the carbohydrate. This tablet is also suitable for testing carbon sources which do not crystallize, such as ethanol or glycerol. To that end a tablet which does not contain carbohydrate is placed in the respective section of the dish, and an appropriate amount of a carbon source in a physiologically tolerable concentration is pipetted onto the tablet. For instance, ethanol or glycerol may be used in 20% by weight solution, in which case the suitable amount will be 50 µl/tablet.

A suitable composition of a tablet containing nitrate, ammonium, or urea for testing nitrogen metabolism is, in percentages by weight, nitrogen source 2.5%, necessary nutrients—e.g. Difco Yeast Carbon Base—3.0%; filler material 85.0%, binder 9.5%.

A preferred basic assortment of tablets for use with a single plate according to the invention consists of tablets for the following purposes: positive control of nitrogen source $(NH_4)_2SO_4$, nitrate, carbohydrate control (without carbohydrate), glucose, maltose, saccharose, inositol, lactose, cellobiose, raffinose, melibiose, erythritol, xylose, dulcitol, trehalose, and urea. Substances may be added to or omitted from this

list as required for the identification of the microorganism in question.

The tablets are preferably put in place on the plate with the aid of a dispenser designed and made especially for this plate. Such a dispenser has a tube for each different type of tablet so that, at a single pressing of the dispenser, each tablet comes into its proper place on the plate. The tablet components are preferably compressed into tablets of a fixed weight and hard enough not to decompose on the culture medium when moistened.

The method described here offers considerable advantages in its preferred embodiment. The amount of material, and the handling of the material, is reduced, as all the tests are performed in the same plate instead of each in a separate test tube. Storage, incubation, and working space is saved, and the tests are more rapid and more reliable. Preparations for the tests are easier and quicker, since the whole plate can be prepared at once and in the same position. Only four culture media are required, instead of one medium for each test tube. The preparation of tablets is simple and quick. No easily contaminated nutrient solutions are required, and so the really troublesome task of preparing and sterilizing these solutions (e.g. sterilization of sugar solutions by filtration) is avoided; tablets need not necessarily be sterilized as long as the preparation procedures are sufficiently aseptic. Drying of filter paper discs, pipetting of solutions, etc., is avoided. The risk of contamination is smaller as the system contains no complete culture medium except one for the morphological test. Plates and tablets can be kept at room temperature. The identification is rapidly performed, and the results can be read after inoculation of the plate.

The following examples illustrate how the invention is applied to identification of yeasts, actinomycetes, and bacteria.

Example 1

A yeast from a clinical sample was cultured pure on Sabouraud agar. A suspension of one or two yeast colonies was prepared in sterile, distilled water. The suspension was inoculated on corn meal agar in one compartment of a plate as shown in the accompanying drawings with a needle by scratching the surface from edge to edge. One end of the scratch was covered with a sterile cover-glass and the other end was left uncovered. Into all other sections of the plate two drops of the same yeast suspension were pipetted with a Pasteur pipette. The yeast suspension was kept at room temperature for future need. The tablets were then applied to the culture media in their respective compartments with the aid of a dispenser designed for that purpose. The plate was placed, right side up, in an incubator at 25°C.

After two and three days the growth on the corn meal agar was microscoped directly in the plate, using 700x magnification and bright field illumination. It was found that the yeast formed hyphae, but chlamydospores and arthrospores did not occur. The other tests gave the following results: After three days' incubation, the positive nitrogen control was positive. The yeast grew on the medium, and the colour of the medium was yellow, as it should always be. The nitrate test was negative, i.e. the colour of the medium was yellow. The carbohydrate control had, as it should, the original dark green colour. Of the sugars glucose, maltose, saccharose, xylose and trehalose were positive; other sugars and urea were negative.

The results indicated that the yeast was either *Candida neoformans* or *Candida parapsilosis*. By a fermentation test it was confirmed that the yeast was *C. parapsilosis*.

Example 2

From the milk of a cow suffering from chronic mastitis, a yeast was isolated and cultured pure. For identification of the yeast, the method described in Example 1 was used. The preparatory measures were the same as in Example 1.

For reading of the results, the microbial growth was microscoped directly on the corn meal agar after two days' incubation. The yeast formed hyphae and pseudohyphae, and arthrospores were formed from the hyphal tips by fragmentation. Nitrogen and carbohydrate controls gave the right reactions, which showed that the plate and the sample were fit for use.

The nitrate test was negative. Of the sugars glucose, lactose, xylose and dulcitol were positive; maltose, saccharose, inositol, cellobiose, raffinose, melibiose, erythritol and trehalose were slightly positive. Urea was positive.

Example 3

A yeast was isolated from tainted orange juice and cultured pure. The yeast was identified by the method of Example 1. The preparatory measures were also the same as in Example 1.

The yeast formed pseudohyphae on corn meal agar. Other results seen after four days: The controls were alright. The nitrate test was positive. Of the sugars glucose, maltose, saccharose, cellobiose, raffinose, erythritol and trehalose were clearly positive; xylose was slightly positive; inositol, lactose, melibiose and dulcitol were negative. Urea was positive.

The yeast was therefore identified as *Hansenula anomala*.

Example 4

The ability of *Escherichia coli* bacteria (serogroup 0149) to assimilate carbohydrates and reduce nitrate as well as their urease production were studied with the identification system according to the invention.

E. coli was incubated overnight in a nutrient broth at 37°C. The bacterial growth was isolated from the medium by centrifugation and suspended to original volume in sterile, distilled water. As in the identification of yeasts, one drop of the suspension was pipetted to each culture medium in a plate according to the invention after

which the respective tablets were added in the manner explained in Example 1. The plates were incubated overnight at 37°C. The results were read as in the identification of yeasts.

E. coli (serogroup 0149) reduced the nitrate to nitrite (red colour upon addition of a drop of sulfanilic acid and alphanaphthylamine). It did not form urease; it did assimilate glucose, maltose, lactose, raffinose, meli-biose, xylose and trehalose, but not saccharose, inositol, cellobiose, erythritol or dulcitol. The positive and negative reactions were clear and corresponded to those described in the literature.

The system is suitable for studying the properties mentioned above, on the basis of which *Escherichia coli*, and particularly Enterobacteriaceae strains, are identified.

Example 5

An identification system according to the invention was used for identifying Streptomyces strains by studying their ability to utilize carbohydrates and nitrate and to produce urease.

Of a culture of streptomycete isolated from the mud of a lake bottom and kept on sodium caseinate agar slanting surface, a suspension was made in sterile, distilled water, as described above for yeasts. A drop of the suspension was pipetted to each agar surface in a plate according to the invention, and the corresponding tablets were added in the manner described in Example 1. The plate was kept for ten days at 25°C, after which the results were read.

The strain under study grew on corn meal agar, forming hyphae and small green colonies. The strain utilized nitrate effectively, but the hydrolyzation of urea was poor. Furthermore, the strain assimilated all the twelve carbohydrates, i.e. glucose, maltose, saccharose, inositol, lactose, cellobiose, raffinose, melibiose, erythritol, xylose, dulcitol and trehalose.

The carbohydrate and nitrate tests were very clearly positive.

In one embodiment the invention may be regarded as a method for the identification of microorganisms by the use of carbohydrate and nitrogen metabolism tests, the urea test and by studying the morphology, all the tests being performed in a handy container with a plurality of compartments, one compartment of which is used for the morphological test and contains the only complete culture medium, while the other compartments contain various buffered or unbuffered solid agar media, with or without an indicator for the carbohydrate and nitrogen metabolism tests and the urea test, which method saves time and room, is easy to perform, guarantees better stability (preservability) and makes a more versatile use possible by being applicable to the identification of a very wide variety of microorganisms and being applicable to the performance of a very wide variety of carbohydrate and nitrogen metabolism tests merely by changing the constituents of the tablets placed on the solid media in the container with the plurality of compartments, the method being characterized in that the solid media for the carbohydrate and nitrogen metabolism tests and the urea test do not contain the nutrients necessary for the growth of microorganisms and that after inoculation with the microorganism there are placed on these solid media tablets which do or do not contain the carbohydrate, nitrogen or urea substances to be tested, all the other nutrients necessary for the growth of the microorganisms and pharmacologically acceptable excipient(s) (fillers) and binder(s).

Claims

1. A device for use in the identification of microorganisms by studying their growth under different circumstances, comprising a plate subdivided into several isolated compartments at least some of which contain a solid medium on which a microorganism to be identified can grow after the addition of one or more nutrients, whereby the effect of adding different possible nutrients for microorganism metabolism can be studied.

2. A device as claimed in claim 1 wherein at least one of the compartments contains a solid medium containing all the nutrients necessary for microorganism growth, whereby the morphology of the microorganism can be studied.

3. A device as claimed in claim 1 or 2 wherein one or more of the solid media lacking one or more nutrients contain an indicator for indicating the results of the study on the effect of adding a particular candidate nutrient.

4. A device as claimed in any of claims 1 to 3 wherein the solid medium is a buffered or unbuffered agar medium.

5. A kit for use in the identification of microorganisms comprising a device as claimed in any of claims 1 to 4 and a plurality of tablets each containing one or more nutrients and/or candidate nutrients for the growth of the microorganism, optionally a binder and/or a filler and optionally an indicator for indicating the results of the study on the effect of adding a particular candidate nutrient.

6. A method of identifying a microorganism employing a kit as claimed in claim 5, comprising inoculating the solid media in the compartments with the microorganism to be identified; studying the morphology of the microorganism growing in at least one of the compartments in the presence of all the necessary nutrients for its growth; adding the tablets to compartments containing solid media lacking one or more nutrients, the tablets being selected so that after their addition and the addition of any nonsolid candidate nutrient each compartment (other than any desired control compartment) has all the necessary nutrients for microorganism growth except that in some compartments a candidate nutrient is present instead of nutrient, a variety of different candidate nutrients being used in different compartments; and studying the effect

on microorganism growth of the candidate nutrients.

7. A method as claimed in claim 6 wherein the candidate nutrients comprise at least one candidate nutrient for studying the carbohydrate metabolism of the microorganism and at least one candidate nutrient for studying the nitrogen metabolism of the microorganism.

8. A method as claimed in claim 7 wherein the studies are confined to the morphology, and carbohydrate metabolism, nitrogen metabolism and urease tests.

9. A method as claimed in claim 7 or 8 wherein for the carbohydrate metabolism test(s) the solid medium in the appropriate compartment(s) is buffered to a pH of 6.5 with 5—10 mM phosphate buffer and bromcresol purple or bromthymol blue is used as indicator of the effect of the candidate carbohydrate nutrient(s).

10. A method as claimed in any of claims 7 to 9 wherein the tablets used in the carbohydrate metabolism test(s) comprise candidate carbohydrate 12.7 to 25.4 wt.%, other nutrient(s) necessary for the growth of the microorganism 4.3 wt.%, filler 60.6 to 74.5 wt.%, and binder 9.5 wt.%.

11. A method as claimed in any of claims 7 to 10 wherein a carbohydrate control test is conducted using a carbohydrate control tablet which does not contain carbohydrate.

12. A method as claimed in any of claims 7 to 11 wherein the candidate carbohydrate nutrients are selected from monosaccharides, disaccharides, oligosaccharides and polysaccharides.

13. A method as claimed in any of claims 7 to 12 wherein a non-solid candidate carbohydrate nutrient is tested in at least one compartment, a carbohydrate control tablet which does not contain carbohydrate being added to that compartment and the non-solid candidate nutrient being added separately.

14. A method as claimed in any of claims 7 to 13 wherein for the nitrogen metabolism test(s) the pH of the solid medium in the appropriate compartment(s) is adjusted to 6.4 and bromthymol blue is used as indicator of the effect of the candidate nitrogen nutrient(s).

15. A method as claimed in any of claims 7 to 14 wherein the tablets used in the nitrogen metabolism tests comprise candidate nitrogen source 2.5 wt.%, other nutrient(s) necessary for the growth of the microorganism 3.0 wt.%, filler 85.0 wt.% and binder 9.5 wt.%.

16. A method as claimed in any of claims 7 to 15 wherein a urease test is conducted, the solid medium in the appropriate compartment has a pH of 6.9, and phenol red is used as an indicator of the result of the urease test.

17. A method as claimed in any of claims 7 to 16 wherein a urease test is conducted and the tablets used for the test comprise urea 2.5 wt.%, other nutrient(s) necessary for the growth of the microorganism 3.0 wt.%, filler 85 wt.% and binder 9.5 wt.%.

18. A method as claimed in any of claims 6 to 17 wherein, for at least one compartment, an indicator for indicating the effect of the candidate nutrients is added to the surface of the solid media after culture and is not present initially in the solid media.

19. A method as claimed in any of claims 6 to 18 wherein the tablets contain cellulose or a cellulose derivative as filler.

20. A method as claimed in any of claims 6 to 19 wherein the tablets contain a plastics product as binder.

21. A method for identifying microorganisms using a device as claimed in claim 1 and carried out substantially as hereinbefore described or exemplified.

22. A device substantially as illustrated in and described with reference to the accompanying drawings.

Printed for Her Majesty's Stationery Office by the Courier Press, Leamington Spa, 1980. Published by the Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.